

## Exine Micromorphology of Orchidinae (Orchidoideae, Orchidaceae): Phylogenetic Constraints or Ecological Influences?

M. R. BARONE LUMAGA<sup>1</sup>, S. COZZOLINO<sup>2</sup> and A. KOCYAN<sup>3,\*</sup>

<sup>1</sup>Orto Botanico and <sup>2</sup>Dipartimento delle Scienze Biologiche, Università di Napoli Federico II, Naples, Italy and

<sup>3</sup>Institute of Systematic Botany, Ludwig Maximilians University, Menzinger Strasse 67, 80638 Munich, Germany

Received: 31 January 2006 Returned for revision: 2 March 2006 Accepted: 13 March 2006 Published electronically: 16 May 2006

• **Background and Aims** Pollen characters have been widely used in defining evolutionary trends in orchids. In recent years, information on pollination biology and phylogenetic patterns within Orchidinae has become available. Hence, the aim of the presented work is to re-evaluate exine micromorphology of Orchidinae in light of recent phylogenetic studies and to test whether pollen micromorphology strictly depends on phylogenetic relationships among species or whether it is influenced by the marked differences in pollination ecology also reported among closely related species.

• **Methods** Pollen sculpturing of 45 species of Orchidinae and related taxa was investigated using scanning electron microscopy. To cover potential intraspecific variation, several accessions of the same species were examined.

• **Key Results** Orchidinae show remarkable variation in exine sculpturing, with a different level of variation within species groups. In some genera, such as *Serapias* (rugulate) and *Ophrys* (psilate to verrucate), intrageneric uniformity corresponds well to a common pollination strategy and close relationships among species. However, little exine variability (psilate–scabrate and scabrate–rugulate) was also found in the genus *Anacamptis* in spite of striking differences in floral architecture and pollination strategies. A larger variety of exine conditions was found in genera *Dactylorhiza* (psilate, psilate–scabrate and reticulate) and *Orchis* s.s. (psilate, reticulate, perforate–rugulate and baculate) where no unequivocal correspondence can be found to either phylogenetic patterns or pollination strategies.

• **Conclusions** Changes in pollen characteristics do not consistently reflect shifts in pollination strategy. A unique trend of exine evolution within Orchidinae is difficult to trace. However, the clades comprising *Anacamptis*, *Neotinea*, *Ophrys* and *Serapias* show psilate to rugulate or scabrate pollen, while that of the clade comprising *Chamorchis*, *Dactylorhiza*, *Gymnadenia*, *Orchis* s.s., *Platanthera*, *Pseudorchis* and *Traunsteinera* ranges from psilate to reticulate. Comparison of the data with exine micromorphology from members of the tribe Orchidoideae and related tribes suggests a possible general trend from reticulate to psilate.

**Key words:** SEM, exine, phylogeny, pollination biology, pollen, orchids.

### INTRODUCTION

Orchidaceae are known for their large diversity in pollen morphology (Schill and Pfeiffer, 1977; Burns-Balogh, 1983; Freudenstein and Rasmussen, 1997). This diversity is present at different levels: variability in packaging of pollen in pollinia, in pollen wall structure, as well as in pollen surface sculpturing (Burns-Balogh and Hesse, 1988). Hence, the structure and shape of pollinia have been used frequently for orchid classification (Burns-Balogh, 1983; Burns-Balogh and Funk, 1986; Rasmussen, 1999, and citations therein).

Pollen characters have been considered useful in defining evolutionary trends in plant families such as Araceae (Grayum, 1986), Callitrichaceae (Osborn *et al.*, 1991, 2001; Osborn and Philbrick, 1994), Hydrocharitaceae (Tanaka *et al.*, 2004), Fabaceae (Papilionoideae; Ferguson and Skvarla, 1982), Scrophulariaceae (*Pedicularis*; Wang *et al.*, 2003) and Orchidaceae (Erdtman, 1960; Caspers and Caspers, 1976; Cronquist, 1981; Burns-Balogh, 1982, 1983; Burns-Balogh and Bernhardt, 1985; Averyanov, 1990; Dressler, 1993; Freudenstein and Rasmussen, 1999). In particular, the exine micromorphology has been frequently used as a reference character in taxonomical and phylogenetic analyses and, in orchids, this

pollen character shows a remarkable diversity among closely related taxa (Schill and Pfeiffer, 1977). However, in some orchid subtribes, such as Disinae, pollen exine sculpture patterns were found to be too variable to allow a distinction even at the species level or, as in Coryciinae, too uniform for taxonomic resolution level (Chesselet and Linder, 1993). Nevertheless, at the generic and subtribal level, the same data produced phylogenetic information (Chesselet and Linder, 1993). Similar contrasting patterns were found by Schill and Pfeiffer (1977) in the genera *Ophrys* and *Orchis* (both of the subtribe Orchidinae): *Ophrys* had a very uniform pollen surface, but *Orchis* (s.l.) showed an astonishing pollen diversity.

There have been several attempts to correlate pollen surface sculpturing and pollen stratification with pollination strategies (Hesse, 2000), and certain general patterns seem to be well established: elaborate pollen sculpturing is often correlated with entomophily, and psilate pollen grains may be characteristic for anemophilous or hypohydrophilous plants (Walker, 1974). Tanaka *et al.* (2004) showed that there is a strong correlation between pollen morphology and pollination mechanisms in Hydrocharitaceae where entomophily seems to be the plesiomorphic state and hypohydrophily is the apomorphic state. Morphologically, the entomophilous pollen grains can be distinguished by the conspicuous spines and a two-layered

\* For correspondence. E-mail kocyana@lrz.uni-muenchen.de

exine, whereas hypohydrophilous pollen showed a reduced exine structure with a smooth surface.

Differences of pollen grains in cases of extremely different pollination mechanisms are functionally understandable and phylogenetically traceable. However, it may be more difficult to trace evolutionary tendencies within plant groups with similar pollination strategies. This was shown by Wang *et al.* (2003) in *Pedicularis* (Scrophulariaceae) with entomophilous pollen. They found a correlation between pollinators and corolla shape, but none with pollen characters. In orchids, the situation is complicated further by the fact that the pollen grains are not directly attached on the pollinator's body. The whole pollen mass (pollinium) is placed on a stalk (caudiculum or stipes) that ends on the viscidium which is responsible for the attachment on the pollinator (Dressler, 1993).

In an attempt to explain the evolution of orchid pollen surfaces, Burns-Balogh (1983) proposed that the pollen surface characteristics of Orchidaceae can be interpreted as the result of reversal processes going from primitive tectate-perforate to the derivate intectate condition with a reversion to a tectate-imperforate condition both in Epidendroideae and in Neottioideae (today in Epidendroideae). Orchidoideae show conditions with tectate imperforate, semitectate or intectate exine and the general absence of the foot layer. The evolution of exine in Orchidoideae as detailed in Burns-Balogh (1983) implicates a series of exine reductions going from a tectate-imperforate condition, with baculae maintaining lateral extensions residual from tectum demolition, to a semitectate-ectate condition with exine globules laying on the endexine. The scheme proposed by Burns-Balogh has been the subject of criticism, however, due to the small sampling within the family (Zavada, 1990; Pridgeon, 1999).

Nevertheless, independently of any evolutionary reconstruction, Orchidoideae have the widest range of pollen features in the orchid family (Hesse and Burns-Balogh, 1984). Pollen grains of orchidoids vary subtly in surface sculpture among species (Schill and Pfeiffer, 1977) but, according to Bateman *et al.* (2003), no clear phylogenetic patterns are evident. In contrast, Pridgeon (1999) stated that the pollen heterogeneity of Orchideae may have promising systematic utility. The main limitation in recognizing a phylogenetic signal in pollen characters of orchids depends on the influence that ecological factors, such as differences in pollination strategies, may have on the pollen morphology in spite of evolutionary affinities among taxa. In fact, often, traits pertaining to floral morphology may be interpreted as the results of pollinator-mediated selection and have more ecological than phylogenetic implications.

Clearly, an independently acquired knowledge of species relationships may help in elucidating correlations between pollen morphology and pollination strategies. In orchids, in particular in Orchidinae, such an attempt is still lacking, but in recent years several independent and largely congruent studies (Bateman *et al.*, 1997, 2003; Pridgeon *et al.*, 1997; Aceto *et al.*, 1999; Cozzolino *et al.*, 2001) defined the patterns of phylogenetic relationships of the subtribe Orchidinae (Orchidoideae) based on nuclear internal transcribed spacer (ITS) sequences. In particular, most members

of the old genus *Orchis* have been split into three related genera: *Anacamptis*, *Neotinea* and *Orchis* (s.s.) (Bateman *et al.*, 1997). These clades found support from karyological data and root tuber characteristics, though, in general, additional morphological synapomorphies defining these clades are still wanted (Bateman *et al.*, 2003).

Often, information on pollination biology of Orchidaceae is relatively scarce as most orchid species are epiphytes living high up on trees, thus making it difficult to observe pollination. Information on pollination is thus more easily accessible in terrestrial orchids. In particular, Orchidinae have a substantial pollination observation record (e.g. van der Cingel, 1995; Pridgeon *et al.*, 2001, and references therein). This makes Orchidinae an ideal candidate for a case study on the correlation of pollen characteristics and pollination biology when phylogenetic patterns are known.

The aim of this study is a re-evaluation of the exine micromorphology, based on newly produced and literature data, of allied members of the subtribe Orchidinae in the light of the recent molecular phylogenetic reconstructions of the group (Bateman *et al.*, 2003). In particular, we are interested in ascertaining whether the variation in pollen micromorphology reflects phylogenetic relationships among species or whether it may be significantly influenced by the striking difference in pollination ecology also found among closely related species (Neiland and Wilcock, 1994; Aceto *et al.*, 1999; Cozzolino *et al.*, 2001).

## MATERIALS AND METHODS

### *Plant material*

Forty-five species of Orchidinae, of which three species were formerly included in Habenariinae (Table 1), have been investigated. Pollinaria were collected from plants cultivated at the Botanical Garden of Naples, Italy or in the wild (see Table 1). To cover intraspecific variation, pollinaria of 2–6 individuals per species were sampled. Sampling was conducted over 3 years. Pollinaria were removed by sticking the viscidium on a small piece of Parafilm pellicle.

### *Taxa classification, pollination biology and pollen nomenclature*

Classification of Orchidinae (including the former Habenariinae) follows those of Bateman *et al.* (1997, 2003), Dressler (1993) and Pridgeon *et al.* (2001). Data regarding orchid pollination are reported in van der Cingel (1995) and reference therein. Pollen nomenclature follows that of the 'Glossary of pollen and spore terminology' (available on-line at <http://www.bio.uu.nl/~palaeo/glossary/glos-int.htm>).

### *Scanning electron microscopy (SEM)*

Pollinia were fixed in FAA (formalin–acetic acid–alcohol 10:5:50), dehydrated in an ethanol series, critical-point dried in liquid CO<sub>2</sub> and sputter-coated with approx. 30 nm of gold. Alternatively, air-dried pollinia were coated

TABLE 1. Species included in this study, collecting sites and exine micromorphology

Species	Provenance	Exine micromorphology
Subtribe Orchidinae		
<i>Anacamptis (Orchis) caspia</i> (Trautv.) Bateman, Pridgeon & Chase	Israel	Scabrate–rugulate
<i>Anacamptis (Orchis) coriophora</i> (L.) Bateman, Pridgeon & Chase	BGN; southern Italy	Psilate–scabrate
<i>Anacamptis (Orchis) laxiflora</i> (Lam.) Bateman, Pridgeon & Chase	BGN; southern Italy	Psilate
<i>Anacamptis (Orchis) longicornu</i> (Poir.) Bateman, Pridgeon & Chase	Sardinia, Sicily	Psilate
<i>Anacamptis (Orchis) morio</i> (L.) Bateman, Pridgeon & Chase	BGN; southern Italy	Psilate–scabrate
<i>Anacamptis (Orchis) papilionacea</i> (L.) Bateman, Pridgeon & Chase	BGN; southern Italy Sardinia; Sicily	Scabrate–rugulate
<i>Anacamptis pyramidalis</i> (L.) L. C. M. Richard	BGN; southern Italy	Perforate–rugulate
<i>Dactylorhiza romana</i> (Seb. et Maur.) Soó	BGN	Psilate to psilate–scabrate
<i>Dactylorhiza saccifera</i> (Brogn) Soó	BGN; southern Italy	Perforate–rugulate
<i>Dactylorhiza sambucina</i> (L.) Soó	BGN; southern Italy	Psilate
<i>Dactylorhiza (Coeloglossum) viridis</i> (L.) Bateman, Pridgeon & Chase	BGN; southern Italy	Reticulate
<i>Gymnadenia conopsea</i> (L.) R. Br.	BGN; southern Italy	Perforate
<i>Himantoglossum hircinum</i> Spreng.	BGN	Rugulate
<i>Himantoglossum (Barlia) robertianum</i> Bateman, Pridgeon & Chase	BGN; southern Italy; Sicily	Psilate
<i>Neotinea (Orchis) lactea</i> (Poir.) Bateman, Pridgeon & Chase	BGN; Sicily	Psilate
<i>Neotinea maculata</i> (Desf.) Stearn	southern Italy	Rugulate
<i>Neotinea (Orchis) tridentata</i> (Scop.) Bateman, Pridgeon & Chase	BGN	Psilate
<i>Neotinea (Orchis) ustulata</i> (L.) Bateman, Pridgeon & Chase	BGN; southern Italy	Psilate
<i>Ophrys apifera</i> Huds.	BGN; southern Italy	Psilate–scabrate to verrucate
<i>Ophrys bertolonii</i> Moretti	BGN	Psilate–scabrate to verrucate
<i>Ophrys fusca</i> Link	BGN	Psilate–scabrate to verrucate
<i>Ophrys lacaitae</i> Lojac.	BGN; southern Italy	Scabrate–verrucate
<i>Ophrys lutea</i> Cav.	BGN; Sicily	Psilate–scabrate
<i>Ophrys sphegodes</i> Mill.	BGN	Psilate
<i>Ophrys tenthredinifera</i> Willd.	BGN; Sicily	Psilate–scabrate to verrucate
<i>Orchis anatolica</i> Boiss.	Israel	Psilate–scabrate
<i>Orchis anthropophora</i> (L.) All.	BGN	Reticulate
<i>Orchis galilaea</i> (Bornmüller & Schulze) Schlechter	Israel	Reticulate
<i>Orchis italica</i> Poir.	BGN	Verrucate
<i>Orchis mascula</i> L.	Sardinia, southern Italy	Psilate
<i>Orchis militaris</i> L.	Northern Italy	Reticulate
<i>Orchis pauciflora</i> Ten.	BGN	Psilate
<i>Orchis provincialis</i> Balb.	BGN	Psilate
<i>Orchis purpurea</i> Huds.	BGN	Reticulate
<i>Orchis quadripunctata</i> Cyrill. ex Ten.	BGN; southern Italy	Perforate–rugulate
<i>Orchis simia</i> Lam.	BGN; southern Italy	Reticulate
<i>Platanthera chlorantha</i> Cust. ex Rchb.	BGN; southern Italy	Psilate–scabrate
<i>Serapias cordigera</i> L.	BGN; southern Italy	Rugulate
<i>Serapias lingua</i> L.	BGN	Rugulate
<i>Serapias orientalis</i> (Greuter) H. Baumann & Künkele	BGN	Rugulate
<i>Serapias parviflora</i> Parl.	BGN	Rugulate
<i>Serapias vomeracea</i> (Burm. f.) Briq.	BGN; southern Italy	Rugulate
Subtribe Habenariinae		
<i>Cynorkis purpurascens</i> Thou.	Botanical Garden, Munich	Rugulate
<i>Habenaria</i> sp. Willd.	Botanical Garden, Zurich	Baculate (pilate)
<i>Herminium monorchis</i> R. Br.	BGN	Baculate (pilate)

BGN: Botanical Garden of Naples, Italy.

with gold. We observed no differences of the investigated structures between critical-point- and air-dried material. To preserve the exine and intine, no acetolysis was carried out (Hesse and Waha, 1989). Specimens were observed under a Cambridge 250Mark3 and under a FEI-Quantas 200 ESEM, at the CISME centre, Università degli Studi di Napoli ‘Federico II’. As pollen characters may vary depending on the position on the massulae (Caspers and Caspers, 1976), we decided to compare only exine structures at the distal massulae ending to achieve maximum comparability (Fig. 1). In addition, we focused on exine structures at the outer surface of the pollen tetrad or massula, respectively, as the exine structure between the pollen grains can be largely reduced (Averyanov, 1990).

## RESULTS AND DISCUSSION

Neither intra- nor interindividual variation in exine micromorphology was observed for the species for which different accessions were examined.

SEM observations of all investigated *Serapias* species showed a substantial uniformity in the genus with a characteristic rugulate exine (Fig. 2; Supplementary Information, available online). This uniformity corresponds to the close phylogenetic relationship among all species and also to the common pollination strategy based on the mimicry of a sleeping hole for solitary insects. The autogamous *S. parviflora* is notable as it shows the same exine pattern as all other allogamous species.



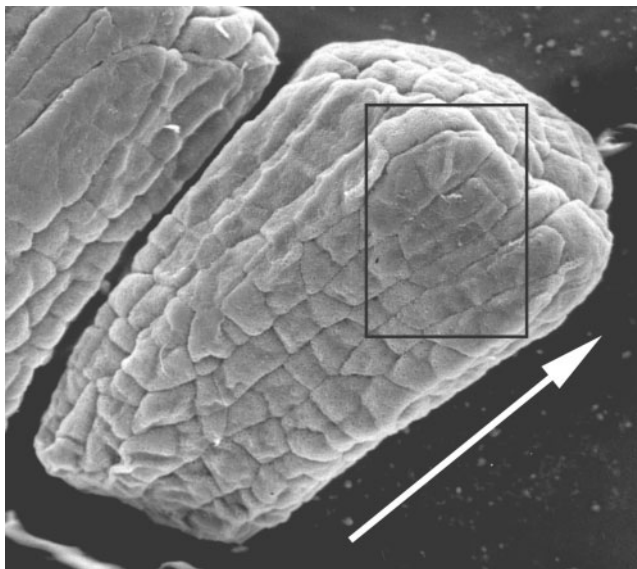


FIG. 1. The rectangle on the massula of *Cynorkis purpurascens* illustrates the approximate position where the exine structures were investigated in this study. The arrow is pointing in the distal direction.

In the sexually deceptive genus *Ophrys*, SEM observations show only small differences in the exine micromorphology of investigated taxa ranging from psilate to scabrate-verrucate exine (Fig. 2; Supplementary Information). In detail: *O. apifera* and *O. bertolonii* show a psilate–scabrate to verrucate exine; in the *O. fusca*–*lutea* complex, *O. fusca* shows a psilate–scabrate to verrucate exine and *O. lutea* shows a psilate–scabrate exine; *O. sphegodes* shows a psilate exine; *O. tenthredinifera* shows a psilate–scabrate to verrucate exine; and *O. lacaitae* shows a scabrate–verrucate exine. Our results are in accordance with the findings of the work of Caspers and Caspers (1976) where a general psilate condition of *Ophrys* pollinia surface was reported. These authors, in their study, also reported the presence of pores. However, if pores were visible, they were mainly from acetolysed pollinia. Caspers and Caspers (1976) even stressed that the observed differences can be detectable on the same massula, or at least in different massulae of the same pollinia. Therefore, they doubted the real usefulness of such sculptural characters for differential diagnosis. Schill and Pfeiffer (1977) pointed out two different types of pollinia surface structures, namely the  $\pm$ verrucose–hamulate type of *Ophrys attica*, *O. bertolonii*, *O. biscutella*, *O. reinholdii*, *O. speculum* and *O. tenthredinifera*, and the psilate–scabrose type of *Ophrys atrata*, *O. fuciflora*, *O. fusca*, *O. kurdica*, *O. parviflora* and *O. scolopax*. However, they also mentioned that some taxa (*Ophrys apifera*, *O. garganica*, *O. lutea* and *O. sphegodes*) represent transitional stages between the states described above. Our current study comprises species of both groups of Schill and Pfeiffer (our *Ophrys lacaitae* was formerly described as *O. fuciflora* ssp. *lacaitae*), but we have not found any significant differences that could confirm their proposed division into two groups. Thus, we are more

inclined to consider these ambiguous exine structures, also observed within the same *Ophrys* species, as intraindividual variations rather than real distinct categories.

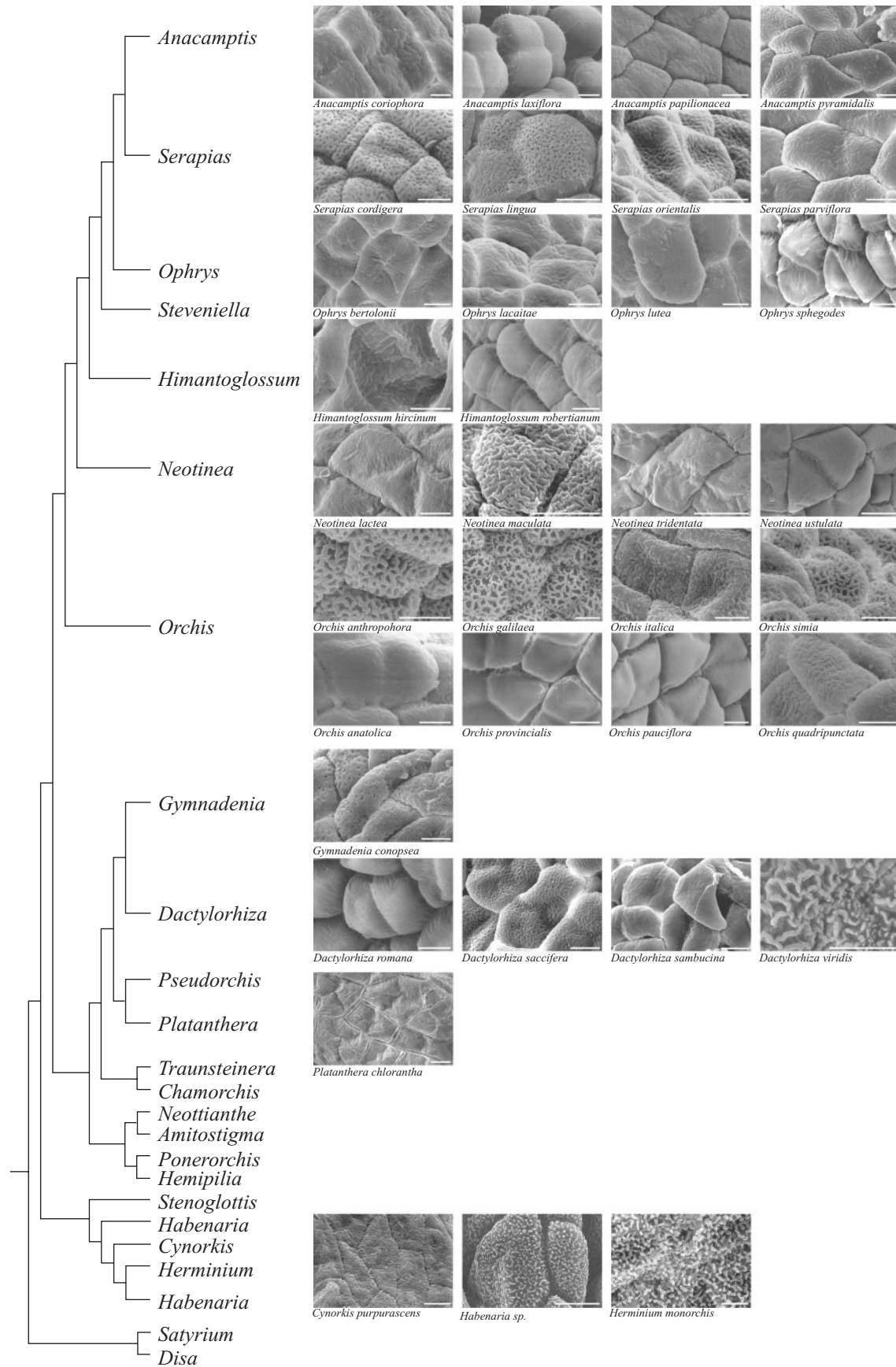
Similarly, little variability has been found in the exine of the genus *Anacamptis*, where the exine reduction leads to a psilate–scabrate to scabrate–rugulate condition (Fig. 2; Supplementary Information). In fact, *A. caspia* and *A. papilionacea* show a scabrate–rugulate exine; *A. coriophora* and *A. morio* show a psilate–scabrate exine; *A. laxiflora* and *A. longicornu* show a psilate exine; and *A. pyramidalis* shows a perforate to rugulate exine.

The low exine variability detected contrasts sharply with the substantial differences in floral architecture and pollination strategies observed within this group: most members of *Anacamptis* have short to long spurs and are nectar cheaters, including the aberrant long-spurred *Anacamptis pyramidalis* that is pollinated by long-tongued day- and night-flying Lepidoptera. Two species, however, are nectar rewarding (represented by *Anacamptis coriophora* in our study). Neither the moth-pollinated (*A. pyramidalis*) nor the nectar-rewarding species (*A. coriophora*) show remarkable differences in their pollen sculpturing. This corresponds well to the phylogenetic position of *A. pyramidalis* and *A. coriophora*, both deeply nested in the *Anacamptis* clade (Bateman *et al.*, 2003).

Interestingly, in the related clade *Himantoglossum* (Fig. 2), *H. hircinum* shows a rugulate exine while *H. (Barlia) robertianum* shows a psilate exine. The two species, in spite of their phylogenetic affinity, are clearly distinct in pollination strategies (van der Cingel, 1995).

In the *Neotinea* clade (Fig. 2), *Neotinea maculata*, that is sister to the rest of *Neotinea* in Bateman *et al.* (2003), is different in exhibiting a rugulate exine surface whereas the remaining investigated species have psilate exine. Our results are similar to those reported by Schill and Pfeiffer (1977). The somewhat isolated position of *N. maculata* can be interpreted in accordance with its pollination strategy: *N. maculata* is self-pollinating (cleistogamous) whereas other *Neotinea* species have allogamous, food deceptive flowers.

More pronounced discontinuity in exine micromorphology is detected in the *Gymnadenia*–*Dactylorhiza* clade (Fig. 2). The ornamentation of the small genus *Gymnadenia* is psilate–perforate to ornate (Schill and Pfeiffer, 1977; Xi *et al.*, 2000; Fig. 2, this study). *Dactylorhiza* (formerly *Coeloglossum*) *viridis* shows a reticulate exine with fragmented muri. The other *Dactylorhiza* species are clearly different, showing a psilate to psilate–scabrate exine. Only *D. saccifera* shows perforate rugulate surfaces. In general, these findings agree with those of Schill and Pfeiffer (1977). Nevertheless, they reported for some northern European taxa (*D. elata*, *D. maculata*, *D. majalis* and *D. traunsteineri*) a verrucose–hamulate sculpturing. Similar to our sculpturing types and those of Schill and Pfeiffer (1977), Averyanow (1990) describes three types of sculpturing in *Dactylorhiza*: psilate–scabrose, verrucose–hamulate and reticulate–fragmentimurate. This latter type was reported only for *D. iberica*. It would be interesting to check whether this species, not examined yet

FIG. 2. Modified ITS phylogeny presented by Bateman *et al.* (2003) with exine sculpturing SEMs arranged accordingly. Scale bars indicate 10 μm.

in available phylogenetic analyses, groups together with *D. viridis*, which shows a similar exine micromorphology.

Current phylogenetic reconstruction of *Dactylorhiza* (Bateman *et al.*, 2003) indicates *Dactylorhiza viridis* as a sister species of a larger clade containing, among others, *D. romana*, *D. sambucina* and *D. saccifera*. This basal position may indicate that pollen of *D. viridis* possesses some basal traits for this *Dactylorhiza* group. However, this scenario is very unlikely because members of the other *Dactylorhiza* clades also have psilate exines (Schill and Pfeiffer, 1977) such as the related *Platanthera chlorantha* that shows a psilate–scabrate exine (Fig. 2). Rather, the reticulate exine condition found in *D. viridis* is more likely to be an autoapomorphy for this species and may reflect the strong ecological shift in its pollination strategy. *Dactylorhiza viridis* offers nectar in a short spur as a reward to a broad range of pollinators (van der Pijl and Dodson, 1966; van der Cingel, 1995) in contrast to the rest of related *Dactylorhiza* species which are food deceptive (Nilsson, 1980; van der Cingel, 1995).

Pollen of rewarding species is expected to be delivered to specific stigmas in a shorter time than pollen of deceptive species because of insect constancy in visiting a rewarding species (Cozzolino and Widmer, 2005). However, it is unknown if a longer permanence of pollinia on the insect body, expected in deceptive orchids, may promote a difference in exine micromorphology in order to prevent excessive pollen dehydration.

In the newly circumscribed genus *Orchis* (s.s.; Fig. 2; Supplementary Information), the formerly monospecific *Orchis* (= *Aceras*) *anthropophora* shows a reticulate–fragmentimurate exine. The closely allied species *O. galilaea*, *O. purpurea*, *O. militaris* and *O. simia* are reticulate, while *O. italica* has a verrucate exine. All these species are short spurred.

Among long-spurred *Orchis* s.s., *O. mascula*, *O. provincialis* and *O. pauciflora* show a psilate exine, the thin-long-spurred *O. quadripunctata* shows a perforate–rugulate exine, while its vicariant taxon, *O. anatolica*, shows a psilate–scabrate exine. These latter two species are expected to have a similar pollination biology, even if detailed studies on their pollination are still lacking (van der Cingel, 1995).

Of all the investigated groups, the *Orchis* s.s. clade is the most divergent and variable in exine condition, ranging from psilate, psilate–scabrate, verrucate, perforate–rugulate to reticulate. A similar diversity was also described by Schill and Pfeiffer (1977) but when considering the old circumscription of the genus *Orchis* s.l. A phylogenetic reconstruction of the exine evolution in the group is difficult as the different exine types are scattered over the ITS cladogram of Bateman *et al.* (2003) (Fig. 2) with even closely allied species such as *O. anatolica* and *O. quadripunctata* showing different exine characters. However, within the genus, some main trends can be recognized. For instance, the long-spurred species group, mainly pollinated by social and solitary long-tongued bees (i.e. *O. mascula*, *O. pauciflora* and *O. provincialis*), are all characterized by psilate exine. On the contrary, the short-spurred *Orchis* species display a reticulate exine with the notable exception of

verrucate exine of *O. italica*. In Bateman *et al.* (2003), *O. anthropophora* is sister to the rest of *Orchis* s.s., and *O. militaris* and *O. simia* are part of a large ‘core’ *Orchis* clade whereas *O. italica* is sister to all *Orchis* (but not to *O. anthropophora*). However, in the phylogenetic reconstruction of Aceto *et al.* (1999) and Cozzolino *et al.* (2001), *O. italica* is sister to all other *Orchis* species (including *O. anthropophora*), and in a strict consensus tree of chloroplast sequence data (A. Widmer and A. Kocyan, unpublished data), *O. anthropophora* is sister to *O. purpurea*, *O. militaris*, *O. simia*, *O. galilaea* and *O. punctulata*. According to these alternative reconstructions, the reticulate condition is a clearly defined morphological character and agrees to a certain extent with pollination. In general, short-spurred *Orchis* s.s. are nectar cheaters pollinated by short-tongued solitary bees, beetles and flies. The only exception is *O. galilaea* that is probably sexually deceptive and attracts males of solitary bees by scent emission (Dafni, 1987).

Due to the large variation in exine morphology, a phylogenetic trend of exine evolution in the subtribe Orchidinae is difficult to trace (Fig. 2). However, one main difference clearly is elucidated: the clade comprising *Anacamptis*, *Himantoglossum*, *Neotinea*, *Ophrys* and *Serapias* shows, in general, psilate to rugulate or scabrate pollen, whereas the clade comprising *Dactylorhiza*, *Gymnadenia*, *Orchis* s.s., *Platanthera*, *Pseudorchis* and *Traunsteinera* (unknown pollen state)/*Chamorchis* (reticulate–fragmentimurate; Schill and Pfeiffer, 1977) has a much wider range of pollen sculpturing, from psilate to reticulate with several intermediate stages.

The former clade, with the notable exception of the *Neotinea* species group, is characterized by a chromosomal number of  $2n = 36$  while the latter has  $2n = 40$  and  $42$  as typical chromosomal numbers (D’Emérico, 2001). Hence, this main dichotomy in pollen micromorphology finds strong correspondence in the phylogenetic reconstruction of Orchidinae proposed by Bateman *et al.* (2003) who, in contrast to the phylogenetic reconstructions of Aceto *et al.* (1999) and Cozzolino *et al.* (2001), suggested *Neotinea* ( $2n = 42$ ) as sister clade of the  $2n = 36$  orchids.

Unambiguous reconstruction of evolutionary trends in the tribe Orchidieae cannot be firmly supported, and our data do not help in disclosing the basal relationship in Orchidinae that are still lacking (see Bateman *et al.*, 2003). Pollen sculpturing data of sister clades comprising *Amitostigma*, *Hemipilia*, *Neottianthe* and *Ponerorchis* give an ambiguous signal. *Hemipilia* has reticulate pollen (Luo, 1999) and *Neottianthe* has a psilate–perforate to reticulate pollen surface (Schill and Pfeiffer, 1977; Xi *et al.*, 1998). *Stenoglottis*, *Herminium*, *Habenaria* or *Disa* pollen micromorphology does not indicate a linear trend: *Stenoglottis* is reticulate–heterobrochate (Schill and Pfeiffer, 1977), *Cynorkis purpurascens* is rugulate (Fig. 2), *Herminium monorchis* is baculate (Fig. 2), some *Habenaria* species are baculate (Fig. 2), hamulate or ornate (Schill and Pfeiffer, 1977), or reticulate or spinulate (Hesse and Burns-Balogh, 1984), and *Disa* species are baculate–psilate, ornate or reticulate (Schill and Pfeiffer, 1977; Chesselet and Linder, 1993).



According to Chase *et al.* (2003), Orchidinae and Disinae are sister to Brownleeinae, all together representing the tribe Orchidieae, which is sister to the monogeneric tribe of Codonorchideae. Plotting pollen characters on their cladogram, it seems possible that there is an evolutionary trend from foveolate to reticulate (Codonorchideae), reticulate (Brownleeinae) to psilate (including intermediate stages to verrucose, scabrate, rugulate and perforate) in Disinae and culminating in the Orchidinae *Anacamptis*–*Himantoglossum*–*Neotinea*–*Ophrys*–*Serapias*–*Steniseiella* clade with the largely psilate (but not reticulate) stage. The reticulate pollen types of the other Orchidinae *Dactylorhiza*, *Gymnadenia*, *Orchis s.s.*, *Platanthera*, *Pseudorchis* and *Trautsteinera*/*Chamorchis* clade (i.e. the  $2n = 40, 42$  clade) should then be reversals from a psilate stage, thus implying a derivate position for these orchids compared with the  $2n = 36$  (namely *Ophrys*, *Serapias*, *Himantoglossum* and *Anacamptis*) plus *Neotinea* clade.

Orchids are considered a paramount example of evolution through floral diversification (Cuzzolino and Widmer, 2005). Before the molecular era, orchid systematics were mainly based on floral morphological traits. However, these traits have turned out to be very homoplastic and thus unsuitable for phylogenetic reconstruction because, as the result of pollinator-mediated selection, they revealed more ecological than phylogenetic implications (Bateman *et al.*, 1997; Aceto *et al.*, 1999; Cuzzolino *et al.*, 2001). If this was true also for the exine structures investigated in the present study, we would expect a larger variation in this trait according to the frequently observed changes in pollination strategies. For instance, for *A. pyramidalis* and *A. coriophora*, species with unique floral characters within the genus *Anacamptis*, different pollen structures can be expected, which was not found in this study. Thus it can be assumed that, at least in some clades, such as in the *Anacamptis*–*Himantoglossum*–*Neotinea*–*Ophrys*–*Serapias*–*Steniseiella* clade, with mostly psilate stage, the pollen sculpturing is more likely to reflect their evolutionary history. In contrast, in some genera such as *Dactylorhiza* and *Orchis s.s.*, species groups characterized by similar pollination biology revealed marked differences in pollen sculpturing. The absence of a univocal relationship between pollen micromorphology and pollination strategies has been confirmed by recent evidence that showed that several species characterized by different pollen sculpturing show a large overlap in pollinator set. Different and unrelated orchid species such as *D. romana*, *A. morio* and *O. mascula*, when growing in sympatry, have been found to adopt a largely overlapping set of pollinator species (Cuzzolino *et al.*, 2005) irrespective of the marked differences in their pollen sculpturing. At the same time, closely related species with presumably identical pollination biology, such as *O. quadripunctata* and *O. anatolica*, have different exine morphology.

These pieces of evidence suggest that a convergent pollination syndrome is not always reflected in a preferential pollen sculpturing and that, at the same time, a shift to a different pollination strategy does not necessarily imply a significant change in pollen micromorphology. In light of this, the finding of species with marked difference in pollen

micromorphology when compared with their close relatives (e.g. *N. maculata*, *D. viridis* and *O. italica*) may also reflect the effects of relaxed selection on this trait or the consequence of different evolutionary constraints of flower topology (such as pleiotropic effects induced, for instance, by the evolution of cleistogamy or by modification of floral parts) rather than the ecological adaptation *per se* to a different pollinator functional group.

## SUPPLEMENTARY INFORMATION

SEM pictures of the following taxa are mentioned in the text but not printed in this article: *Anacamptis caspia*, *Anacamptis morio*, *Anacamptis longicornu*, *Ophrys apifera*, *Ophrys fusca*, *Ophrys tenthredinifera*, *Orchis militaris*, *Orchis mascula*, *Serapias vomeracea*. They are available as Supplementary Information online at <http://www.aob.oxfordjournals.org/>.

## ACKNOWLEDGEMENTS

The authors thank Jasmin Joshi and Alex Widmer for valuable discussions on orchid pollination, and Paolo Grunanger for kindly providing some orchid pollinaria. We would like to thank V. Avolio, S. Giorgio and R. Salatiello of the Naples Botanical Garden for help with plant collection and cultivation, and the PRIN Research Programme for supporting research on orchid biology.

## LITERATURE CITED

- Aceto S, Caputo P, Cuzzolino S, Gaudio L, Moretti A. 1999. Phylogeny and evolution of *Orchis* and allied genera based on ITS DNA variation: morphological gaps and molecular continuity. *Molecular Phylogenetics and Evolution* 13: 67–76.
- Averyanov L.V. 1990. A review of the genus *Dactylorhiza*. In: Arditti J, ed. *Orchid biology, reviews and perspectives* Vol. 5. Portland, OR: Timber Press, 159–206.
- Bateman RM, Pridgeon AM, Chase MW. 1997. Phylogenetics of subtribe Orchidinae (Orchidoideae, Orchidaceae) based on nuclear ITS sequences. 1. Infrageneric relationships and reclassification to achieve monophyly of *Orchis* sensu stricto. *Lindleyana* 12: 113–141.
- Bateman RM, Hollingsworth PM, Preston J, Yi-Bo L, Pridgeon AM, Chase MW. 2003. Molecular phylogenetics and evolution of Orchidinae and selected Habenariinae (Orchidaceae). *Botanical Journal of the Linnean Society* 142: 1–40.
- Burns-Balogh P. 1982. Generic redefinition in subtribe Spiranthinae (Orchidaceae). *American Journal of Botany* 69: 1119–1132.
- Burns-Balogh P. 1983. A theory on the evolution of the exine in Orchidaceae. *American Journal of Botany* 70: 1304–1312.
- Burns-Balogh P, Bernhardt P. 1985. Evolutionary trends in the androecium of the Orchidaceae. *Plant Systematics and Evolution* 149: 119–134.
- Burns-Balogh P, Funk V. 1986. A phylogenetic analysis of the Orchidaceae. *Smithsonian Contribution to Botany* 61: 1–79.
- Burns-Balogh P, Hesse M. 1988. Pollen morphology in the cypripedioid orchids. *Plant Systematics and Evolution* 158: 165–182.
- Caspers N, Caspers L. 1976. Zur Oberflächenskulpturierung der Pollinien mediterraner *Orchis* und *Ophrys* Arten. *Pollen et Spores* 18: 203–215.
- Chase MW, Cameron KM, Barrett RL, Freudenstein JV. 2003. DNA data and Orchidaceae systematics: a new phylogenetic classification. In: Dixon KW, Kell SP, Barrett RL, Cribb PJ, eds. *Orchid conservation*. Kota Kinabalu: Natural History Publications (Borneo), 69–89.
- Chesselet P, Linder HP. 1993. Pollen morphology on the Daseae (Orchidoideae; Orchidaceae). *Grana* 32: 101–110.

- van der Cingel NA. 1995. *An atlas of orchid pollination. European orchids*. Rotterdam: Balkema Publishers.
- Cozzolino S, Widmer A. 2005. Orchid diversity: an evolutionary consequence of deception? *Trends in Ecology and Evolution* 20: 487–494.
- Cozzolino S, Widmer A, Dafni A. 2001. Pollination of tribe Orchideae. In: Pridgeon AM, Cribb PJ, Chase MW, Rasmussen FN, eds. *Genera Orchidacearum. Vol. 2, Orchidoideae, part 1*. Oxford: Oxford University Press, 232–233.
- Cozzolino S, Schiestl FP, Muller A, De Castro O, Nardella AM, Widmer A. 2005. Evidence for pollinator sharing in Mediterranean nectar-mimic orchids: absence of premating barriers? *Proceedings of the Royal Society: B* 272: 1271–1278.
- Cronquist A. 1981. *An integrated system of classification of flowering plants*. New York: Columbia University Press.
- Dafni A. 1987. Pollination in *Orchis* and related genera: evolution from reward to deception. In: Arditti, J, ed. *Orchid biology, reviews and perspectives*. Ithaca and London: Cornell University Press, 79–104.
- D'Emérico S. 2001. Tribe Orchideae cytogenetics. In: Pridgeon AM, Cribb PJ, Chase MW, Rasmussen FN eds. *Genera Orchidacearum. Vol. 2, Orchidoideae, part 1*. Oxford: Oxford University Press, 216–224.
- Dressler R. 1993. *Phylogeny and classification of the orchid family*. Portland: Dioscorides Press.
- Erdtman G. 1960. Pollen walls and angiosperm phylogeny. *Botaniska Notiser* 113: 41–45.
- Ferguson IK, Skvarla JJ. 1982. Pollen morphology in relation to pollinators in Papilionoideae (Leguminosae). *Botanical Journal of the Linnean Society* 84: 183–193.
- Freudenstein JV, Rasmussen FN. 1997. Sectile pollinia and relationships in the Orchidaceae. *Plant Systematics and Evolution* 205: 125–146.
- Freudenstein JV, Rasmussen FN. 1999. What does morphology tell us about orchid relationships?—A cladistic analysis. *American Journal of Botany* 86: 225–248.
- Grayum MH. 1986. Correlations between pollination biology and pollen morphology in the Araceae, with some implications for angiosperm evolution. In: Blackmore S, Ferguson IK, eds. *Pollen and spores. Form and function*. Linnean Society Symposium Series 12. London: Academic Press, 313–327.
- Hesse M. 2000. Pollen wall stratification and pollination. *Plant Systematics and Evolution* 222: 1–17.
- Hesse M, Burns-Balogh P. 1984. Pollen and pollinarium morphology of *Habenaria* (Orchidaceae). *Pollen et Spores* 26: 385–400.
- Hesse M, Waha M. 1989. A new look at the acetolysis method. *Plant Systematics and Evolution* 163: 147–152.
- Luo LB. 1999. *Studies on the orchid genus Hemipilia*. PhD Thesis, Institute of Botany, Chinese Academy of Science, Beijing, China.
- Neiland MRM, Wilcock CC. 1994. Reproductive ecology of European orchids. In: Pridgeon AM, ed. *Proceedings of the 14th World Orchid Conference*. Edinburgh: HMSO, 138–147.
- Nilsson LA. 1980. The pollination ecology of *Dactylorhiza sambucina* (Orchidaceae). *Botaniska Notiser* 133: 367–385.
- Osborn JM, Taylor TN, Schneider EL. 1991. Pollen morphology and ultrastructure of the Cabombaceae: correlations with pollination biology. *American Journal of Botany* 78: 1367–1378.
- Osborn JM, Philbrick CT. 1994. Comparative pollen structure and pollination biology in the Callitrichaceae. *Acta Botanica Gallica* 141: 257–266.
- Osborn JM, El-Ghazaly G, Cooper RL. 2001. Development of the exineless pollen wall in *Callitriche truncata* (Callichitraceae) and the evolution of underwater pollination. *Plant Systematics and Evolution* 228: 81–87.
- van der Pijl L, Dodson CH. 1966. *Orchid flowers. Their pollination and evolution*. Coral Gables: University of Miami Press.
- Pridgeon AM. 1999. Palynology. In: Pridgeon AM, Cribb PJ, Chase MW, Rasmussen FN, eds. *Genera Orchidacearum. Vol. 1*. Oxford: Oxford University Press, 33–37.
- Pridgeon AM, Bateman RM, Cox AV, Hapeman JR, Chase MW. 1997. Phylogenetics of subtribe Orchidinae (Orchidoideae, Orchidaceae) based on nuclear ITS sequences. 1. Intergeneric relationships and polyphyly of *Orchis* sensu lato. *Lindleyana* 12: 89–109.
- Pridgeon AM, Cribb PJ, Chase MW, Rasmussen FN. 2001. *Genera Orchidacearum. Vol. 2, Orchidoideae, part 1*. Oxford: Oxford University Press.
- Rasmussen FN. 1999. The development of orchid classification. In: Pridgeon AM, Cribb PJ, Chase MW, Rasmussen FN, eds. *Genera Orchidacearum. Vol. 1*. Oxford: Oxford University Press, 3–12.
- Schill R, Pfeiffer W. 1977. Untersuchungen an Orchideenpollinien unter besonderer Berücksichtigung ihrer Feinskulpturen. *Pollen et Spores* 19: 5–118.
- Tanaka N, Uehara K, Murata J. 2004. Correlation between pollen morphology and pollination mechanisms in the Hydrocharitaceae. *Journal of Plant Research* 117: 265–276.
- Walker JW. 1974. Evolution of exine structure in the pollen of primitive angiosperms. *American Journal of Botany* 61: 891–902.
- Wang H, Mill RR, Blackmore S. 2003. Pollen morphology and infrageneric evolutionary relationships in some Chinese species of *Pedicularis* (Scrophulariaceae). *Plant Systematics and Evolution* 237: 1–17.
- Xi Y-Z, Lang K-Y, Hu Y-S. 1998. Pollen morphology of *Neottianthe* (Orchidaceae) and its taxonomic significance. *Acta Phytotaxonomica Siniaca* 36: 496–502.
- Xi Y-Z, Lang K-Y, Hu Y-S. 2000. Pollen morphology of *Gymnadenia* R. Br. (Orchidaceae) and its taxonomic significance. *Acta Phytotaxonomica Siniaca* 38: 137–140.
- Zavada MS. 1990. A contribution to the study of pollen wall ultrastructure of orchid pollinia. *Annals of the Missouri Botanical Garden* 77: 785–801.